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In re Patent Application of

TICKLE, et al.

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Filed: October 23, 2003

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For: CRYSTAL STRUCTURE OF CYTOCHROME P450

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December 23, 2003

Commissioner for Patents
P.O. Box 1450
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Sir:

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It is respectfully requested that this application be given the benefit of the foreign filing date under the provisions of 35 U.S.C. §119 of the following, a certified copy of which is submitted herewith:

Application No.	Country of Origin	Filed
0108214.8	Great Britain	02 April 2001
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Respectfully submitted,

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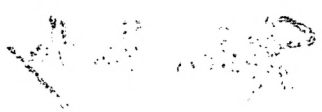

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P01/7700 0.00-0108214.8

1. Your reference — AHB/CP5924360

2. Patent application number *(The Patent Office will fill in this part)* 02 APR 2001 **0108214.8**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
Patents ADP number *(if you know it)*

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4. Title of the invention — METHODS OF PROTEIN PURIFICATION AND USES THEREOF

5. Name of your agent *(if you have one)* — MEWBURN ELLIS

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Patents ADP number *(if you know it)* — 109006

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11. I/We request the grant of a patent on the basis of this application.

Signature: Martin Gras

Date: 2 April 2001

12. Name and daytime telephone number of person to contact in the United Kingdom: ADRIAN H. BRASNETT 020 7240 4405

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Methods of Protein Purification and Uses Thereof

The present invention relates to methods for preparing cytochrome P450 molecules, particularly in a form suitable for crystallization.

Cytochrome P450s are a very large and complex gene superfamily of hemeproteins that metabolise physiologically important compounds in many species of microorganisms, plants and animals. Cytochrome P450s are important in the oxidative, peroxidative and reductive metabolism of numerous and diverse endogenous compounds such as steroids, bile, fatty acids, prostaglandines, leukotrienes, retinoids and lipid. Many of these enzymes also metabolise a wide range of xenobiotics including drugs, environmental compounds and pollutants.

Mammalian cytochrome P450s are 50-55 kDa heme-thiolate proteins that are found in either the mitochondrial inner membrane (type I) or in the endoplasmic reticulum network of the cell (type II). The type II or microsomal enzymes are integral membrane proteins anchored by an N-terminal transmembrane spanning α-helix. The bulk of the enzyme faces the cytoplasmic surface of the lipid bilayer as opposed to the lumen. For activity, cytochrome P450s require other membranous enzymatic components including the flavoprotein NADPH-cytochrome P450 oxidoreductase and, in some cases, cytochrome b5. A single cytochrome P450 oxidoreductase supports the activity of all the mammalian microsomal enzymes by interacting directly with the P450s and transferring the required two electrons from NADPH. Cytochrome b5 is necessary for increasing electron transfer for certain P450 isoforms and specific substrates. Cytochrome P450s are able to incorporate one of the two oxygen atoms of an O_2 molecule into a broad variety of substrates with concomitant reduction of the other oxygen atom by two electrons to H_2O. Cytochrome P450

are known to catalyze hydroxylations, epoxidation, N-, S-, and O-dealkylations, N-oxidations, sulfoxidations, dehalogenations, and other reactions.

Homo sapiens has 17 cytochrome P450 gene families and 42 subfamilies that total more than 50 sequenced isoforms. Cytochrome P450s from families 1, 2 and 3 constitute the major pathways for drug metabolism. Many drugs rely on hepatic metabolism by cytochrome P450s for clearance from the circulation and for pharmacological inactivation. Conversely, some drugs have to be converted in the body to their pharmacologically active metabolites by P450s. It is estimated that 50% of all known drugs are modified by P450s and many promising lead compounds are terminated in the development phase due to their interaction with a cytochrome P450. One of the greatest problems in drug discovery is the prediction of the role of cytochrome P450s on the metabolism or modification drug leads. Early detection of metabolic problems associated with a chemical lead series is a paramount importance for the drug industry. Obtaining crystal structures of the main human drug metabolising cytochrome P450s would be highly valuable for drug design as this would provide detailed informations on how P450 enzymes recognise drug molecules and the mode of drug binding. This in turn would allow to drug companies to develop strategies to modify metabolic clearance and decrease the attrition rates of compounds in development.

The genes of the P450 superfamily have been categorised by Nelson et al (Pharmacogenetics, 6;1-42, 1996), the disclosure of which is incorporated herein by reference, who proposed a systematic nomeclature for the family members. This nomenclature is used widely in the art, and is adopted herein (the prefix of "CYP" is omitted when referring to subfamily

groups). Nelson et al provide cross-references to database sequence entries for P450 sequences.

To date 8 cytochrome P450 structures have been solved by X-ray crystallography and are available in the public domain. Five structures correspond to bacterial cytochrome P450s: P450cam (CYP101 Poulos *et al.*, 1985, *J. Biol. Chem.*, 260, 16122), the hemeprotein domain of P450BM3 (CYP102 , Ravichandran *et al.*, 1993, *Science*, 261, 731), P450terp (CYP108, Hasemann *et al.*, 1994, *J. Mol. Biol.* 236, 1169), P450eryF (CYP107A1, Cupp-Vickery and Poulos, 1995, *Nature* Struct. Biol. 2, 144), P450 14α-sterol demethylase (CYP51, Podust *et al.*,,2001, *Proc. Natl. Acad. Sci. USA*, 98, 3068). The structure of cytochrome P450nor was obtained from the denitrifying fungus Fusarium oxysporum (Shimizu *et al.* 2000, *J. Inorg. Biochem.* 81, 191). Recently the crystal structure of a thermophilic cytochrome P450 (CYP119) from Archaeon sulfolobus solfataricus has been reported (Yano *et al.*, 2000, *J. Biol. Chem.* 275, 31086-31092). The eighth structure is the rabbit 2C5 isoform, the first and only structure of a mammalian cytochrome P450 (Williams *et al.* 2000, *Mol. Cell.* 5, 121-131). All of the cytochrome P450s, whose structures have been solved, were expressed in *E. coli*. The reason why so far, the mammalian cytochrome P450s have been particularly difficult to be crystallize, compared to their bacterial counterparts, resides in the nature of these proteins. The bacterial cytochrome P450s are soluble whereas the mammalian P450s are integral membrane proteins. The mammalian cytochrome P450s are inserted in the membrane of the endoplasmic reticulum by a short, highly hydrophobic N-terminal segment that acts as a non cleavable signal sequence for insertion into the membrane. The remainder of the mammalian cytochrome P450 protein is globular structure that protrudes into the cytoplasmic space.

Most of mammalian cytochrome P450s are located in the liver, but other organs and tissues have high concentrations of certain cytochrome P450s, including the intestinal wall, lung, kidney, adrenal cortex and nasal epithelium. While a number of cytochrome P450 enzymes have been successfully isolated from mammalian microsomes, purification from tissue is not trivial. Purification is complicated by low cytochrome P450 availability in human tissues, the limited yields, difficulties to isolate highly related isoforms that share a high amino acid identity, the hydrophobic nature of these proteins, the use of detergent and, at times, the loss of activity during the purification process. The method of purification from tissues requires the preparation of the microsomal membranes, the extraction of the membrane associated cytochrome P450s with detergent, successive purification steps and the removal of the detergent in the final purification. The cytochrome P450s that have been isolated following this procedure have exhibited a high tendency to aggregate, low solubility and poor monodispersity that makes X-ray crystallography attempts impossible without the use of detergents.

Structural studies on mammalian cytochrome P450s definitively requires the combination of heterologous expression systems that allow expression of single cytochrome P450s at high concentration and modifications in their sequences to improve the solubility and the behaviour of these proteins in solution. Mammalian cytochrome P450s are now routinely expressed as recombinant protein in many different systems, including mammalian cell culture, yeast, baculovirus and bacteria (refer to Methods in Enzymology, Vol. 206, Academic Press, 1991). The latter system has been employed successfully for the expression of a number of microsomal P450 enzymes, in large amounts and at low costs for biophysical or structural studies (Barnes *et al.*, 1991, *Proc. Natl. acad. Sci. USA* 88 , 5597-5601). Expression of

several full-length mammalian cytochrome P450s in *E. coli* are now well documented in the literature (Fisher *et al.* 1992, *FASEB J.* 6, 759-764; Richardson et al. 1995, *Arch Biochem. Biophys.* 323, 87-96, Gillam *et al.* 1995, *Arch. Biochem. Biophys.* 317, 374-384). Modification of a few residues at the N-terminus of the protein has been described as advantageous to optimise expression in *E. coli*. The change of the second codon to an alanine residue is commonly used. However, the physical properties of these full-length cytochrome P450s are very similar to those described for the cytochrome P450s isolated from mammalian tissues, and therefore, are useless for structural studies.

Is it now well recognized that the deletion of the N-terminal membrane anchor region in conjunction with the high level of expression achieved in heterologous systems could provide the means to obtain preparative amounts of soluble mammalian cytochrome P450s for X-ray crystallography. This is based in the assumption that by eliminating the N-terminal hydrophobic signal sequence, a functional cytochrome P450 form lacking its microsomal membrane anchor is produced. Such proteins would be expected to be less hydrophobic, probably even soluble, and presumably more amenable to X-ray crystallography.

Several attempts to produce soluble mammalian cytochrome P450s by removing the N-terminus have been reported in the literature by different groups. Similar N-terminal deletions of cytochrome P450s have been used in each case, though the methods of purification that have been applied and the results obtained differ greatly. There is thus a need for an improved and more generally applicable method of purification.

Cytochrome P450 N-terminal deletion has been successfully applied by Johnson's group to solve the X-ray crystallographic

structure of the first mammalian P450 (Willaims *et al.* 2000, *Mol. Cell.* 5, 121-131). This group (Wachenfelt *et al.*, 1997, *Arch. Biochem. Biophys.* 339, 107-114; Cosme *et al.*, 2000, *J. Biol. Chem.* 275, 2545-2553) have expressed the rabbit 2C3 and 2C5 isoforms in *E. coli* with residues 3-21 at the N-terminus deleted. The results indicate that the truncated proteins exhibit a salt-dependent association with the cell membrane suggesting that the removal of the membrane spanning domain converts these intrinsic membrane proteins to peripherical membrane proteins that could be purified without the use of detergent. The resulting purified proteins were predominantly dimers and tetramers. Difficulties in the crystallization of the rabbit cytochrome P450 2C5 were overcome by the combination of the removal of the membrane spanning sequence and engineering in a region that is believed to be involved in the membrane association.

Kempf *et al.* (Kempf *et al.*, 1995, *Arch. Biochem. Biophys.*, 321, 277-288) have analyzed the expression in *E. coli* of a series of constructs of the human cytochrome P450 2D6 where the N-terminal 25 amino acids were replaced with a hexa-histidine following the N-terminal Met codon. The protein was solubilized from the membrane in high salt and then purified in presence, or absence, of the detergent $C_{12}E_9$ using Ni^{2+} -chelate affinity chromatography followed by purification using DEAE-Sephacel and Hydroxylapatite resin. Their results have shown that in the absence of detergents, the protein exhibits a strong tendency to aggregate into multimeric states. Greater than 50 % of the protein is highly aggregated. The protein could be dissociated to a mononer if the nonionic detergent NP40 was used during the purification and kept in the sample. Three columns were required to achieve a high purity and final yield of 20%.

Gillam *et al.* (Gillam *et al.*, 1995, *Arch. Biochem. Biophys.* 319, 540-550). have examined a series of N-terminal modified constructs of human cytochrome P450 2D6 for expression in *E. coli*. Although, several of these produced some cytochrome P450 2D6, there was considerable variation in the expression rate. The best construct involved the removal of the N-terminal hydrophobic region. The expressed cytochrome P450s were first, extracted from membranes in presence of detergent Triton X114 and then purified using a flavodoxin affinity column and a hydroxylapatite in presence of detergent. The detergent was eliminated in the final preparation by dialysis or by extensive washing. Their results have shown that even deleted of their membrane anchoring sequence, the proteins still bind the membrane and need to be extracted with detergents. The overall recovery of cytochrome P450 2D6 is less than 20%. No data on the aggregation state of the protein was presented.

Pernecky *et al.* (Pernecky *et al. Proc. Natl. Acad. Sci. USA,* 1993, 90, 2651-2655) have expressed the rabbit N-truncated forms of cytochrome P450 2B4 and 2E1 and several chimeras in *E. coli*. The proteins were extracted with 1% n-octyl β D glucopyranoside from the bacterial lysate and successively purified on GSH-Sepharose column and treated with thrombin in order to release the GST tag. Finally, residual detergent was removed from the preparation on a hydroxylapatite column. The results have shown that in absence of detergent, the truncated cytochrome P450 2E1 is still largely aggregated (pentamers) and cytochrome P450 2B4 was a mixture of high-molecular-weight aggregates with an average octomeric size. Addition of detergent was required to convert the protein to a monomeric form.

Larson *et al.* (Larson *et al.*, 1991, *J. Biol. Chem*, 266, 7321-7324) found that rabbit cytochrome P450 2E1 expressed in *E. coli* without amino acids 3-29, like the full-length cytochrome P450,

was predominantly in the membrane fraction. The inability to liberate the truncated cytochrome P450 from membranes with 0.1M Na_2CO_3, pH11, provides evidence for an integral association of the shortened cytochrome P450 2E1 with the membrane. The protein was partially purified after it solubilization from the *E. coli* membrane using n-octyl β D glucopyranoside as detergent.

Saraga *et al.* (Saraga et al., 1993, *Arch. Biochem. Biophys.* 304, 272-278) have expressed a truncated form of the bovine microsomal 17β-hydroxylase cytochrome P450 (P450c17) lacking its N-terminal hydrophobic signal anchor sequence (residues 2-17). The results indicate that the truncated cytochrome P450 is primarily associated with the membrane. No information on this protein was presented.

Disclosure of the Invention.

Despite the efforts such as those of the prior art cited above, there is still a need to develop methods for the purification of P450s from recombinant host cells in a reliable manner, and in yields which allow crystals of the proteins to be formed in sufficient quantity and quality to be amenable for X-ray crystallographic studies.

The present invention aims to overcome the difficulties of the prior art by providing for efficient isolation and purification of P450s. In our studies we have found that a problem with the prior art is that P450 proteins tend to aggregate during their isolation and purification. We believe that one reason for this, which has not previously been identified, is the perceived need in the art to resuspend host cells expressing P450s in a low salt buffer prior to lysis.

In addition, the prior art also teaches that after lysis it is necessary to recover the membrane fraction (usually by high speed ultracentrifugation of the cell lysate) of the host cells before bringing the cells into contact with a detergent in order to remove the P450s from the membrane fraction.

These steps reduce yield of the P450. We have now found a means to recover P450 from a cell lysate without the need to recover a separate membrane fraction. The process uses a high salt buffer at an earlier stage in the recovery process, and provides for a high recovery of protein in a non-aggregated state.

Thus in a first aspect, the invention provides a method for the purification of P450, wherein said method comprises:

(a) expressing in a host cell culture a cytochrome P450 molecule;

(b) recovering said cells from said culture and suspending said cells in a 200 mM to 1000 mM salt buffer;

(c) lysing said cells and removing cell debris to provide a high-salt lysate;

(d) adding to said lysate from 0.015% to 1.2% v/v of a detergent to provide a high-salt-detergent lysate; and

(e) recovering said P450 from said lysate.

The recovery step generally involves affinity purification of the P450 from the high salt-detergent lysate, since the presence of the high salt rules out the alternative of an ionic exchange purification step.

However, once the P450 has been affinity purified, the salt needs to be removed in order to allow additional purification of the product so that crystallization can be performed. In the prior art, salt removal is typically performed by dialysis. However, we have found that this process, which removes salt

gradually over a 12 - 24 hour period, causes aggregation and denaturation of the P450s and thus is undesirable. We have found that rapid desalting alleviates this problem to a significant degree.

Thus in a further aspect, step (e) above may be performed by:

(e(i)) binding said P450 to an affinity support;

(e(ii)) rinsing said support in a high-salt-detergent wash;

(e(iii)) removing said P450 in a high-salt-detergent buffer to provide a P450-high-salt-detergent preparation; and

(f) removing salt from said preparation by size-exclusion chromatography to provide a P450-low-salt preparation.

The above steps e(i)-(iii) maintain the P450 in a high-salt and detergent buffer throughout the initial stages of the purification process, which aids the recovery of the P450.

The preparation may be subject to additional purification and cleaning procedures, such as cation exchange chromatography, optionally followed by further size-exclusion chromatography to obtain a more purified preparation of protein.

The protein preparation recovered may be crystallized, for example by the hanging drop method or other conventional techniques in the art, and subject to x-ray crystallographic analysis. In a further aspect, the invention thus provides a crystal of a P450 protein molecule, and a method obtaining the crystal structure of a P450 molecule which comprises subjecting said crystal to x-ray diffraction, and analysing the diffraction pattern obtained to determine the 3-dimensional coordinates of the atoms of said P450.

Description of the Drawings.

Figure 1 shows the N-terminal sequences of 2C9 and 2C19, together with truncations which remove the membrane-inserting N-terminal region.

Figure 2 is a table showing the recovery of 2C9 at the various stages of purification.

Figure 3 is a table showing the recovery of 2C19 at the various stages of purification.

Detailed Description of the Invention.

Cytochrome P450

We believe that the novel purification process of the invention will be broadly applicable to most cytochrome P450 proteins, since the process is directed to providing at an early stage in purification these proteins in a soluble disaggregated form.

Cytochrome P450 families of interest include the families CYP1, CYP2, CYP3, CYP4, CYP5, CYP6, CYP7A, CYP7B, CYP8, CYP9, CYP10, CYP11, CYP12, CYP13, CYP14, CYP15, CYP16, CYP17, CYP18, CYP19, CYP21, CYP24, CYP27, CYP51 and CYP52. Of these the families of cytochromes of vertebrates are of particular interest, namely the families CYP1, CYP2, CYP3, CYP4, CYP5, CYP7A, CYP7B, CYP8, CYP11, CYP17, CYP19, CYP21, CYP24, CYP27 and CYP51.

Of these, the CYP subfamilies include the 1A (particularly 1A1 and 1A2), 1B, 2A, 2B, 2C, 2D (particularly 2D6), 2E (particularly 2E1), 2F, 3A (particularly 3A4), 4A, 4B, 4C, 4D, 4E, 5A, 11A, 11B subfamilies.

Human cytochrome P450 genes are of particular interest, including human genes of the above families and subfamilies. The sequences of the genes are available on a number of public databases, including SwissProt. Human P450s include 1A1 (SwissProt P04798 (all following entries SwissProt unless indicated otherwise)), 1A2 (P05177), 1B1 (Genbank/EMBL U03688), 2A6 (P11509), 2A7 (P20853), 2B6 (P20813), 2D6 (P10635), 2E1 (P05181), 2F1 (P24903), 3A3 (P05184), 3A4 (P08684), 3A5 (P20815), 4B1 (P13584), 5A1 (P24557), 4A11 (Genbank/EMBL L04751), 4F2 (Genbank/EMBL U02388), 4F3 (Q08477), 7A (P22680), 11A1 (P05108), 11B1 (P15538), 11B2 (P19099), 17 (P05093), 19 (P11511), 21 (P04033), 27 (Q02318) and 51 (Genbank/EMBL U23942).

Non-human homologues of the above members are also of interest, i.e. non-human proteins from the same sub-families as those mentioned herein which have a high degree (>70% sequence identity) to the proteins mentioned above. Other mammalian P450s include dog P450s such as 2D15 (Genbank/EMBL D17397) and 3A12 (P24463), and rat such as 3A1 (P04800).

A particular group of proteins of interest are the human 2C subfamily. There are four human 2C cytochrome P450 molecules, 2C8, 2C9, 2C18 and 2C19. The sequences of these are available in the art, for example from a number of database sources cited in Nelson et al, 1996, *ibid.* This includes the SwissProt database, wherein:

P450	SwissProt
2C8	P10632
2C9	P11712
2C18	P33260
2C19	P33261

In the present invention, it will be understood that reference to cytochromes includes both the full length membrane bound

sequences, as well as these proteins in which there is a deletion of the N-terminal segment which inserts the protein into the membrane. Such truncated proteins have been widely generated in the art for the purposes of enhancing purification of P450s. See for example von Wachenfeldt et al, *Archives Biochem. Biophys.* 339, 107-114, 1997.

In addition, the P450 may comprise a polypeptide tag allowing for affinity purification. A polyhistidine tag, having from 4 to 10 histidine residues is suitable for this purpose. Other tags include a glutathione S-transferase tag, a streptavadin tag, a MBP (maltose binding protein) tag, a CBD (cellulose binding domain) tag, or an HA tag or the like which can be bound by an antibody. Tags of this type are widely available in the art from academic and commercial sources.

The tag may be at the C- or N-terminus of the P450.

Host cell

The host cell in which the P450 is expressed is any suitable host cell which a person of skill in the art wishes to use as a matter of experimental convenience. Cytochrome P450 molecules have been widely expressed in *E.coli*, and there are numerous vector systems for this host cell which may be used.

Other host cells include yeast, e.g. *S.cerevisiae*, insect or mammalian, e.g. CHO, cells. Expression systems for these and many other host cell types are widely available in the art.

Host cells may be constructed so that the P450 is expressed constitutively, or is induced.

Once the cells have been cultured to express P450, they may be recovered by standard techniques available in the art. A

convenient means is to recover the cells by low-speed centrifugation such that the cells are pelleted intact.

The process of the present invention is suitable for batch cell culture, and batches of cells from 100 ml to 10 litres can be conveniently handled by current laboratory equipment, though larger batches are not excluded.

Salt buffer

The salt buffer used to suspend the cells is a salt which is readily soluble in the 200 - 1000 mM range. Preferably the salt is a potassium or sodium salt of an anion. Desirably the anion may be chloride or phosphate. Potassium phosphate is particularly preferred.

A preferred salt concentration is around 500 mM, e.g. 500 $\pm$ 50 mM.

The buffer will be maintained at a pH range of from 6.5 to 8.0, preferably from 7.0 to 7.4. The buffer may contain other reagents used conventionally in the art for protein purification, such as glycerol, β-mercaptoethanol, DNase, pH buffering agents, and protease inhibitors.

Cell lysis

Cells may be lysed by physical means, such as sonication or in a French press, such that the cell walls are broken and the contents of the cells dispersed in the salt buffer. To achieve this in a French press, this may be operated at 10,000 to 20,000 psi.

Cell debris is removed (for example by low-speed centrifugation at about 10,000 g or the like; i.e. such that any whole cells are pelleted but not the membrane fraction). The debris (e.g.

pelleted cells) may be subject to a further round of lysis, and the debris-free lysate from this further round combined with the lysate obtained previously.

The lysate is then ready to use directly in the next stage of the process, without the need to isolate a membrane fraction by ultracentrifugation.

Detergent

Once the lysate has been obtained, it is desirable that the detergent be added to the lysate as soon as possible, taking account of the constraints of the experimental set up. This will mean that the lysate is brought into contact with the detergent within 1 hour, preferably within 30 minutes or less of the preparation of the debris-free lysate.

The detergents which may be used are those conventionally used in the art of molecular and cell biology for the recovery and processing of biological materials. A large number of different types of detergents are available for this purpose. Many of these detergents are those of a molecular weight range of from about 350 to 1000, such as from 400 to 800. They include anionic surfactants such as cholic acid or salts thereof (e.g. the sodium salt) and deoxycholic acid or salts thereof (e.g. the sodium salt) as well as zwitterionic surfactants such as CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate).

A particularly preferred class of detergents are non-ionic detergents. There are a wide variety of non-ionic detergents available in the art. Non-ionic detergents include octyl-β-D-glucopyranoside and ethers, such as C2-10 alkylphenol ethers, of polylethylene glycol. Such compounds may be of a molecular weight range of 500 - 800 Da, and include Nonident™ P40, IGEPAL

CA630, and Triton™ X-100, and the like, which are commercially available.

The detergent is added to provide a concentration of from 0.015% to 1.2% v/v of detergent in the lysate. The amount of detergent added is preferably in the range of 0.1 to 0.5%, more preferably about 0.2 to 0.4%, such as about 0.3%.

It is added in a volume so that desirably, the concentration of salt does not decrease by more than 10%.

Recovery of P450

We have found that the above high-salt-detergent lysate prepared in accordance with the invention provides for the recovery of P450 protein in monodisperse form at a level much higher than experienced to date in the art. As mentioned above, the presence of the high salt buffer rules out an ionic exchange chromatography step, but affinity purification may be performed as the next step.

Affinity purification may take the form of providing an affinity support matix in which a ligand for the P450 is attached. The support may be a resin, a bead (e.g. glass or polymer such as polystyrene), a magnetic bead, or the like. Where the P450 contains a tag, the ligand will be cognate to the tag, e.g. Ni-NTA for a histidine tag, biotin for a strepavadin tag, etc. The ligand may also be an antibody, either to an epitope tag such as an HA tag, or to an epitope of the P450.

The lysate is brought into contact with the affinity support under conditions for the P450 to bind to the support. After binding, the support is then rinsed. The rinse buffer should be a high-salt-detergent buffer, which may be the same or different to the lysate buffer. Preferably it is the same. If different,

it will still have concentrations of salt and detergent as specified above.

After rinsing, the P450 is removed from the support. This may be done by packing the support into a column, and eluting the P450 using a high-salt-detergent buffer (either the same or different, as in the preceding paragraph) which is modified to provide for removal of the P450 from its ligand. For example, for Ni-NTA, the buffer may contain histidine at a sufficient excess concentration to displace the His tag of the P450. Analogous competitors may be used to for other types of tags.

Desalting

The recovered P450 is then desalted by a rapid desalting process. We have found that a size exclusion column may be used for this purpose, with a flow rate such that the P450 is separated from the high salt concentration within 10 minutes. The P450 is then recovered by elution through the column with a low salt buffer.

While not wishing to be bound by any one particular theory, we believe that whereas gradual desalting by, for example, dialysis, leads to aggregation and denaturation of P450, the rapid desalting process reduces aggregation to a significant degree.

The low salt buffer is preferably a similar salt to the high salt buffer described above, e.g. a sodium or potassium salt such as a chloride or phosphate, with potassium phosphate again being preferred. By "low salt", it is meant less than 20 mM, and preferably about 10 mM. At this stage, it is not necessary to maintain detergent in the buffer.

Further purification

It is desirable that after the desalting step, the preparation is subject to further purification promptly, i.e. without storage or freezing of the sample. This can be achieved by applying the desalted eluate directly to a further purification column. If not, the eluate from the desalting process is collected and applied within 1 hour to the column. A number of techniques are known as such in the art for the further purification or concentration of protein preparations, and examples of these are outlined in the accompanying examples. They include weak cation exchange columns, such as carboxy-methyl-Sepharose™, BioRex™70, carboxymethyl-Biogel™, and the like, which may be used to further remove detergent, and optionally followed by a size exclusion column, e.g Superose™, Superdex™, Sephacryl™, and the like.

Crystal formation

A number of methods are known as such in the art for obtaining protein crystals. Conveniently, the final protein is concentrated to 20-40 mg/ml in 10-100 mM potassium phosphate with high salt (e.g. 500 mM NaCl or KCl) by using concentration devices which are commercially available.

Crystallization of the protein is set up by the 2 µl hanging drop method and the protein is crystallized by vapour diffusion at 5-25 degrees C against a range of buffer compositions. Crystals may be prepared using a Hampton Screening kit, Poly-ethylene glycol (PEG)/ion screens, PEG grid, Ammonium sulphate grid, PEG/ammonium sulphate grid or the like.

The invention is illustrated by the following examples.

Expression vector

The expression vector pCWOri+, provided by Prof.F. W. Dahlquist, University of Oregon , Eugene, Oregon, was used to express the truncated human cytochrome P450s in the E. coli strain XL1 Blue (Stratagene). Cytochrome P450 cDNAs, modified at their 5'terminus, were introduced at the NdeI/SalI cloning sites in the polylinker of the pCWOri+ vector. Residues 2-29 of the native N terminus of human cytochrome P450s 2C9 and 2C19 that constitute the trans-membrane domain were substituted by the motive AKKTSSKGR. An alanine was introduced as second codon in order to improve the protein expression. A four histidine tag was inserted at the 3' terminus to facilitate the purification of the proteins in high salt buffers.

Bacteria expression

A single ampicillin resistant colony of XL1 blue cells was grown overnight at 37 °C in Terrific Broth (TB) with shaking to near saturation and used to inoculate fresh TB media. Bacteria were grown to an OD600nm =0.4 in TB broth containing 100 µg of ampicillin per ml at 37 °C at 200 rpm. The temperature was shifted to 30 °C and the haem precursor delta aminolevulinic acid was added 15 min prior induction with 1 mM IPTG. The bacterial culture was continued under gentle agitation (175 rpm) at 30 °C for 48 to 72 hours.

Protein purification

The cells were pelleted at 10000 xg for 10 min and resuspended in 500 mM KPi, pH7.4, 20 % glycerol, 10 mM mercaptoethanol, 1:1000 dilution of protease inhibitor cocktail (Calbiochem), 0.1 mg/ml DNase 1 and 5 mM $MgSO_4$. The final volume is 50ml per L culture.

The cells were lysed by passing through a Constant Systems Cell Homogeniser at 15000 psi. The cell debris was then removed by

centrifugation at 10000 xg at 4°C for 30 min. Cell debris was resuspended and the lysis procedure repeated. The lysates are then pooled.

Detergent IGEPAL CA630 (Sigma) was added drop by drop to the lysate at a final concentration of 0.3% (v/v) and the lysate was incubated with previously washed NiNTA resin (Qiagen) overnight at 4°C, using agitation. The NiNTA resin was pelleted by centrifugation at 2000 xg for 2min at 4°C and washed several times with 10 volumes of 500 mM KPi, pH7.4, 20% glycerol, 10 mM mercaptoethanol, 50 mM glycine, 1:1000 dilution of protease inhibitor cocktail, 0.3%(v/v) IGEPAL CA630 by centrifugation at 2000 xg for 2min at 4°C. The resin was washed with 10 resin volumes of 500 mM KPi, pH7.4, 20% glycerol, 10 mM mercaptoethanol, 7.5 mM Histidine, 1:1000 dilution protease inhibitors, 0.3% IGEPAL CA630 by centrifugation as described above. Finally, the resin was packed into a column at 4 °C and the cytochrome P450 eluted with 500 mM KPi, pH7.4, 20 % glycerol, 10 mM mercaptoethanol, 100 mM Histidine, 1:1000 dilution of protease inhibitor cocktail, 0.3%(v/v) IGEPAL CA630.

The cytochrome P450 obtained from the NiNTA column was quickly desalted (<10min) into 10 mM KPi, pH 7.4, 20% glycerol, 0.2 mM DTT, 1mM EDTA using a HiPrep 26/10 desalting column (Pharmacia) on the AKTA FPLC system (Pharmacia), at a flow rate of 5ml/min and collecting 16 ml fractions. The desalted cytochrome P450 was directly applied to a CM Sepharose column (Pharmacia), previously equilibrated with 10 mM KPi, pH 7.4, 20% glycerol, 0.2 mM DTT, 1 mM EDTA. The following step elution protocol is then run on the AKTA FPLC system; wash with 20 column volumes of 10mM KPi, pH 7.4, 20% glycerol, 0.2mM DTT, 1mM EDTA, 75 mM KCl in order to remove any trace of detergent and eluted with the above buffer with KCl concentration increased to 500 mM. The

tables in Figures 2 and 3 show the recovery of 2C9 and 2C19 respectively through the above process.

The P450 fractions were concentrated using a Centriprep or Centricon 30. The P450 sample was applied on the top of a Superose 610/30 gel filtration column (Pharmacia) and eluted at 0.2ml/min with buffer containing 100 mM KPi, pH7.4, 300 mM KCl, 20% glycerol, 0.2 mM DTT. The protein was collected and concentrated up to 40 mg/ml using a centricon for crystallization assays.

Quality assays

The quality of the final preparation was evaluated by:

SDS polyacrylamide gel electrophoresis was performed using commercial gels (Nugen) followed by CBB staining according to the manufacturer's instructions. The purity as estimated by scanning a digital image of a gel was estimated at least 95%.

Gel filtration chromatography using a Superose 6 HR10-30 column (Pharmacia) was performed to assess the aggregation state. The fractionation range for this column is $5x10^3$ to $5x10^6$ Da and is thus well adapted to the resolution of large complexes The column was eluted at 0.2ml/min with buffer containing 100 mM KPi, pH7.4, 300 mM KCl, 20% glycerol, 0.2 mM DTT. 0.2ml protein samples at a concentration of approximately 40 mg/ml were used. Absorbance at 280 nm was monitored and the peak was collected and analyzed using dynamic light scattering.

Light scattering.

Samples (0.2 ml) were analysed by DLS in fluorimeter quartz cells at 90° using laser radiation at 830.3 nm. Data was collected using a log correlator with variable expansion spanning a wide dynamic range. All measurements were performed

at 20 °C with samples collected immediately from the gel filtration column. Runs were usually on average of 10 runs of 10 sec each. To obtain an estimation of the molecular weight, we used a frictional ratio of 1.26 and a partial specific volume of 0.726.

Samples prepared using our new method of purification possessed a good solubility and an absence of significant aggregation as shown by:

- the ratio of far channel extrapolation and measured average scattering was always between 0.999 and 1.003.
- the average count rate did not vary significantly, with approximately 1% standard deviation.
- analysis of the autocorrelation function using bi exponential fitting showed that 2C9 had an estimated Mr of approximately 180 kDa and that 2C19 had an estimated Mr of approximately 160 kDa i.e. both are oligomers composed of no more than four subunits.
- good stability of the samples (over 24h) at 20°C.

Samples prepared by published protocols showed signs of a severe aggregation:

- large fluctuations of the scattered light intensity, with a standard deviation of more than 10%.
- analysis of the autocorrelation function showed very slow exponential decay an indication of the presence of large aggregates (Mr > 10^6 Da), composed of a large number of P450 subunits. These samples also show a high degree of polydispersity.
- samples also showed further aggregation as a function of time.

These signs of severe aggregation in samples prepared by published methods were still present after sample filtration through 20 nm diameter pores or centrifugation at 200,000g for 30 min.

Functionality assays

Activity assays on P450 2C9 and 2C19 were performed using a fluorescent method using LS spectrometer (Perkin Elmer Instruments).

Twenty pmoles of P450 2C9 were reconstituted with 0.1 unit of purified human oxidoreductase in presence of 100 µM of substrate methoxy-4-(trifluoromethyl)-coumarin, a NADPH regenerating system that includes 1.3 mM $NADP^+$, 3.3 mM Glucose-6-phosphate and 0.1 unit glucose-6-phosphate dehydrogenase in 300 µl final volume of 25 mM KPi, pH7.4, 3.3 mM $MgCl_2$. Incubations were performed at 37 °C for several minutes and 7-hydroxy -4-(trifluoromethyl)-coumarin was used as metabolite standard to determinate the metabolic rate. The excitation and emission wavelengths used were respectively 409 and 530nm. The activity of 2C9 was determined to be 0.94 ± 0.09 nmol/min/nmol P450.

Activity assays on P450 2C19 were performed using 20 pmoles of P450 2C9 and reconstituted with 0.1 unit of purified human oxidoreductase in presence of 10 µM of substrate - dibenzylfluorescein, a NADPH regenerating system that includes 1.3 mM $NADP^+$, 3.3 mM Glucose-6-phosphate and 0.1 unit glucose-6-phosphate dehydrogenase in 300 µl final volume of 25 mM KPi, pH7.4, 3.3 mM $MgCl_2$ at 37 °C. Hydroxy-dibenzylfluoresceinwas used as metabolite standard. The excitation and emission wavelengths used were respectively 485 and 538nm. The activity of 2C19 was determined to be 1.27 ± 0.06 nmol/min/nmol P450.

Crystallization

Crystallization of P450 2C19 was achieved at 20 mg/ml protein against 0.1 M Hepes, pH6.0, 1.6 M ammonium sulphate, 2.5% PEG 6000. Crystals grew over a two week period as dark brown needles from an initial precipitate.

Crystallization of 2C9 was obtained at 20 mg/ml in 200 mM ammonium sulphate, 100 mM MES, pH 6.0, 5-30% PEG 6000. Small crystals of 2C9 appeared over two weeks as chunky brown blocks.

CLAIMS

1. A method for the purification of a cytochrome P450, wherein said method comprises:

(a) expressing in a host cell culture a cytochrome P450 molecule;

(b) recovering said cells from said culture and suspending said cells in a 200 mM to 1000 mM salt buffer;

(c) lysing said cells and removing cell debris to provide a high-salt lysate;

(d) adding to said lysate from 0.015 to 1.2% v/v of a detergent to provide a high-salt-detergent lysate; and

(e) recovering said P450 from said lysate.

2. A method according to claim 1 wherein step (e) is above may be performed by:

(e(i)) binding said P450 to an affinity support;

(e(ii)) rinsing said support in a high-salt-detergent wash;

(e(iii)) removing said P450 in a high-salt-detergent buffer to provide a P450-high-salt-detergent preparation; and

(f) removing salt from said preparation by size-exclusion chromatography to provide a P450-low-salt preparation.

3. A method according to claim 1 or 2 wherein the P450 comprises a deletion in its N-terminal membrane inserting element.

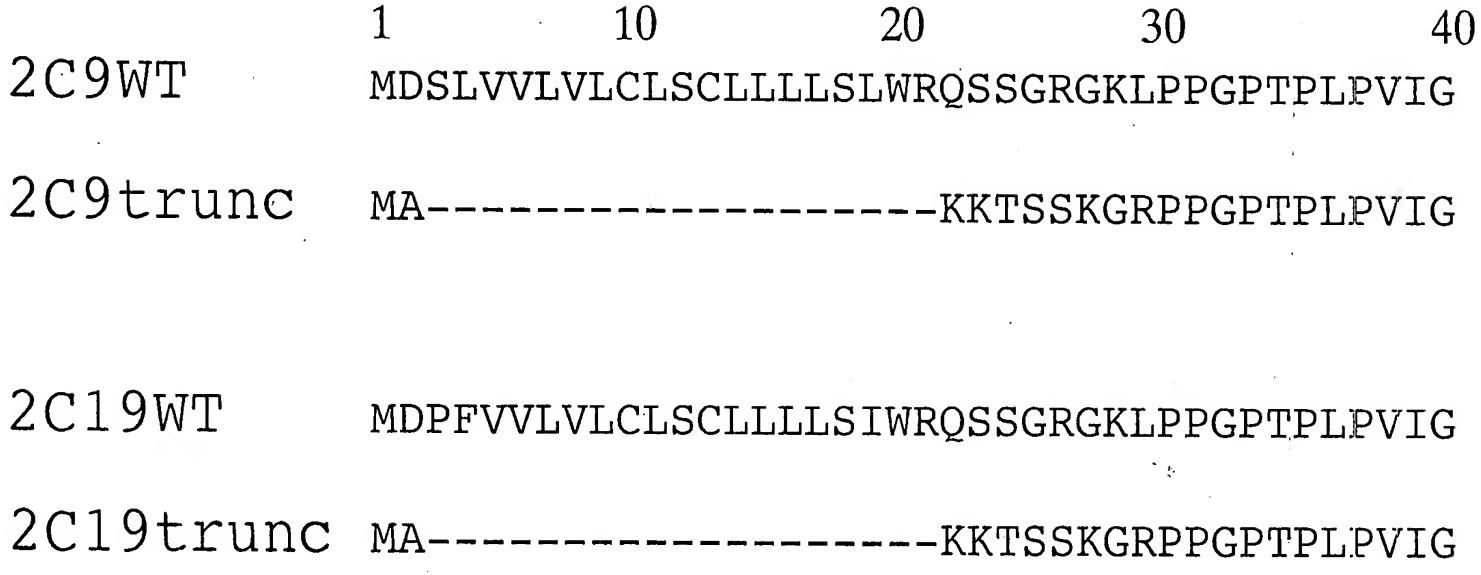
4. A method according to any one of the preceding claims wherein the P450 carries a polyhistidine tag.

5. A method according to any one of the preceding claims wherein the P450 is 2C9 or 2C19.

6. A method according to any one of the preceding claims which further comprises crystallizing the P450.

7. A crystal of a cytochrome P450.

8. A method for determining the crystal structure of a cytochrome P450 which comprises preparing a crystal according to the method of claim 6, subjecting the crystal to x-ray diffraction, and analysing the diffraction pattern obtained to determine the 3-dimensional coordinates of the atoms of said P450.


1 10 20 30 40
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2C9trunc MA----------KKTSSKGRPPGPTPLPVIG
2C19WT MDPFVVLVLCLSCLLLLSIWRQSSGRGKLPPGPTPLPVIG
2C19trunc MA----------KKTSSKGRPPGPTPLPVIG

FIG. 1

Fig. 2

Purification table for 2C9

Purification stage	nmoles 2C19	% recovery
Lysate	708	100
NiNTA Pool	534	75.4
CM Sepharose Flow Through	6.7	0.95
CM Sepharose Pool	566	80.0

Fig. 3

Purification table for 2C19

Purification stage	nmoles 2C19	% recovery
Lysate	847	100
NiNTA 7.5mM Histidine Wash	130	15.0
NiNTA Pool	842	99.0
CM Sepharose Flow Through	7.0	0.9
CM Sepharose Pool	653	77.0